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Detection of Chimerism and Early Engraftment After Allogeneic Peripheral Blood Stem Cell or Bone Marrow Transplantation by Short Tandem Repeats

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Chimerism can be monitored after HLA-matched allogeneic bone marrow transplantation (BMT) or allogeneic peripheral blood stem cell transplantation (PBSCT) by detecting polymorphisms in short tandem repeats (STR). The purpose of our study was to document early complete chimerism in BMT and PBSCT recipients using STR, and to determine whether the initial WBC recovery correlated with the days required to attain complete chimerism. A total of 5 patients (2 PBSCT and 3 BMT) were followed by STR after transplantation. Peripheral blood obtained prior to transplantation was used to determine the 2 most informative STR probes for each donor/recipient pair. STR were amplified by polymerase chain reaction (PCR) with 8 commercial probes, and PCR products were visualized with silver staining. Peripheral blood was evaluated daily post-transplantation for WBC counts and to identify the presence of mixed or full chimerism by STR. The sensitivity of the STR technique varied from 0.05 to 1%, depending on the probe. Full chimerism was documented between day 9 and 14 in PBSCT recipients and on day 14 and 16 in BMT recipients. The initial rise in WBC occurred within 3 days of the onset of full chimerism, indicating that full chimerism is a more sensitive indicator of early engraftment. Periodic recipient monitoring using STR after complete chimerism identifies those patients who revert to mixed chimeras. The STR method may be useful in future studies to determine the significance of early engraftment and the clinical implications of sustained complete chimerism or mixed chimerism.

Key words: chimerism, HLA, allogeneic bone marrow transplantation, short tandem repeats, polymerase chain reaction

INTRODUCTION

Allogeneic peripheral blood stem cell transplantation (PBSCT) and bone marrow transplantation (BMT) are important treatment modalities for many hematologic diseases. Successful outcome may be associated with the development of donor chimerism, consequently it is of interest to examine chimerism early post-transplantation. Many genetic markers are available to document chimerism, and they vary with respect to sensitivity and how frequently they are informative [1]. Engraftment can be inferred from the recovery of peripheral blood counts, but rising cell counts may not always predict the attainment of full donor chimerism. Methods that have been used to monitor allogeneic bone marrow engraftment include cytogenetics, erythrocyte and HLA typing, and DNA sequence polymorphisms. Cytogenetics is only useful if an autosome or sex chromosome difference is present. Erythrocyte typing is generally not useful until 4 or more months post-transplantation [1]. HLA typing is not helpful in the majority of allogeneic transplants as most are performed between HLA-identical, related pairs. DNA polymorphisms can be detected with high sensitivity by using polymerase chain reaction (PCR) to amplify hypervariable regions of human DNA. DNA amplification by PCR allows analysis of DNA sequences even when the WBC count is less than 0.1 K/mm^3, as commonly found post-transplantation [2].

PCR amplification of minisatellite (10–30 base pairs) or variable number of tandem repeats (VNTR) has been

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used by several investigators to evaluate chimerism after BMT [2–5]. Short Tandem Repeats (STR) or microsatellites are repetitive sequences of 3 to 7 base pairs of DNA, and they can also be used to document engraftment [6]. Alleles are differentiated and named by the number of copies of repeat sequences contained within amplified regions. High levels of heterozygosity are found for the STR loci, due to the large number of possible alleles and the distribution of alleles in the population.

The purpose of this study was to follow changes in chimerism in BMT and PBSCT recipients over time using a PCR-based, nonradioactive technique for detecting STR with 8 commercially available probes. Other objectives of this study were to determine whether the day of the initial rise in WBC count would coincide with the onset of engraftment, and whether the chimeric state (full or mixed) post-transplantation would correlate with the clinical course.

MATERIALS AND METHODS

Patients

Five consecutive HLA matched sibling donor-recipient pairs who underwent allogeneic BMT or PBSCT were monitored by STR. Two patients had non-Hodgkin's lymphoma, and one each with acute myelogenous leukemia, acute lymphoblastic leukemia, and multiple myeloma. The PBSCT recipients received T cell depleted peripheral blood stem cells from their HLA identical siblings. All patients were transplanted at the UCSD Medical Center and received preoperative conditioning with fractionated total body irradiation (12–14 Gy) and cyclophosphamide (120 mg/Kg body weight).

This study was approved by the Human Subjects Committee of the University of California, San Diego, La Jolla, California.

Specimens

Peripheral venous blood samples in EDTA or ACD were drawn before transplantation from the donor and recipients. After transplantation, samples were obtained daily from recipients until complete chimerism was observed by STR or at the clinician's discretion. After full chimerism was attained, monthly samples were drawn for patient follow-up. WBC count was determined on a Cell-dyn (Abbott Diagnostics, Abbott Park, IL) from samples drawn at the same time as specimens for STR determination. The rise in WBC was defined as when the patient's WBC recovered from the neutrophil nadir with a sustained rise of WBC $\geq$ 0.1 K/mm^3. Mononuclear cells were isolated using Isoprep lymphocyte isolation media (Robbins Scientific, Sunnyvale, CA). DNA was extracted by salting out with Bio-20 DR DNA Extraction Kit (Bio-Synthesis, Lewisville, TX).

PCR-STR

The PCR master mix components consisted of 5 U/μl Taq Polymerase (Perkin-Elmer Cetus, Irvine, CA), Primer Pair (Gene Print STR Systems, Promega, Madison, WI), STR 10× buffer, and sterile water. Primer pairs at 8 loci were used in this system including CSF1P0, F13A01, F13B, FESFPS, LPL, TH01, vWF, and TPOX. Twenty-five nanograms of patient DNA (in 2.5 μl volume) was added to 22.5 μl of the PCR master mix in each tube. The tubes were placed into the Thermal Cycler (Perkin-Elmer Cetus) and denatured for 2 min at 96°C. Thermocycling conditions for the first 10 cycles were denaturing at 94°C for 1 min, annealing at 60 or 64°C (depending on the primer pairs) for 1 min, and extension at 70°C for 1.5 min. The next 20 cycles were similar with the exception of the denaturing temperature which was changed to 90°C for 1 min. Positive and negative controls were included to detect contamination and to ensure integrity of the system. The positive control was K562 DNA which was provided in the genePrint STR Systems kit (Promega).

Electrophoresis and Visualization

A 2% agarose gel prestained with ethidium bromide was loaded with 8 μl of PCR product and 2 μl loading buffer. The gel was run at 100 V for approximately 1 h. DNA amplification was confirmed by the presence of a band of the appropriate size in each PCR reaction with the exception of the negative controls. Four percent polyacrylamide gels were prepared by combining 16 mL of SequaGel, 74 mL of SequaGel Diluent, and 10 mL of SequaGel Buffer concentrate (National Diagnostics, Atlanta, GA), while stirring with a stir bar. Eight hundred microliters of 10% ammonium persulfate and 40 μL of TEMED (Gibco-BRL, Gaithersburg, MD) were then added while stirring the solution. The acrylamide solution was poured between glass plates and stored overnight. The gel was loaded with PCR samples mixed with STR 2X Loading Solution (GenePrint STR Systems, Promega). Electrophoresis was performed with Sequencing Gel Electrophoresis Apparatus (Gibco-BRL Model S2) at 70 Watts for approximately 2 hours. DNA was visualized with DNA Silver Staining System. The gel was exposed with DNA Electrophoresis Duplication Film (Promega) for documentation and interpretation.

Sensitivity Study

The sensitivity of each primer pair for the 8 loci was determined by mixing DNA from 2 patients, varying the minor population from 0.05 to 1% of the major population. The sensitivity was determined as the lowest concentration of DNA that produced a visible STR band on a silver-stained polyacrylamide gel.

TABLE I. Probe Sensitivity to Detect a Small Population of DNA

Probes	Sensitivity (%)
TPOX, CSF1PO, FESFPS, vWF, LPL	0.05
THO1, F13A01	0.25
F13B	1

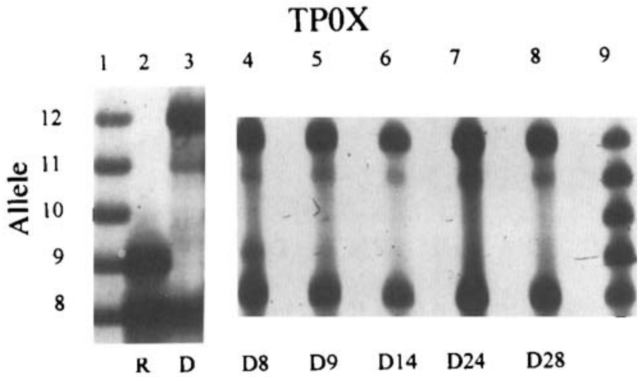

Fig. 1. Documentation of engraftment in patient 1. The ladder of alleles for the TPOX locus is in lanes 1 and 9. The pre-transplantation recipient (R) and donor (D) alleles are in lanes 2 and 3 respectively. Lanes 4–8 show recipient alleles post-transplantation days 8, 9, 14, 24, and 28. On days 8 and 9 both donor and recipient alleles are present, while on days 14, 24, and 28, only donor alleles are present (allele 9 is absent) indicating full chimerism.

Engraftment Monitoring

Initial screening was performed on all donor/recipients with the 8 primer pairs. Two most informative loci were chosen for each pair for engraftment monitoring. Chimerism post-transplantation was determined by the presence of donor- or recipient-specific STR bands. The presence of donor- and recipient-specific bands indicated mixed chimerism, while the presence of donor bands only in recipient's blood indicated full chimerism.

RESULTS

Sensitivity Study

Probe sensitivity varied from 0.05 to 1%. The sensitivities were 0.05% for TPOX, CSF1P0, FESFPS, vWF, and LPL; 0.25% for TH01 and F13A01; and 1% for F13B (Table I).

Detection of Engraftment

At least two informative loci were found among the 8 tested probes for each of the 5 donor/recipient pairs. Figures 1 through 4 demonstrate the change from mixed to full chimerism in four of the donor/recipient pairs. Following the bone marrow transplantation, both donor- and recipient-specific bands (mixed chimerism) were observed. After complete chimerism was attained, only donor bands were present. BMT patients became complete chimeras on day 14 and 16 post-transplantation, while the PBSCT recipients were completely engrafted on day 9 and between day 9 and 14 (no blood was drawn days 10–13). Table II shows patient characteristics and day of engraftment post-transplantation.

Relationship of Rising WBC Count to Complete Chimerism

Figure 5 shows the relationship between the onset of full chimerism and the rise in total WBC count. All recipient WBC counts were less than 0.1 K/mm^3 after chemotherapy and total body irradiation, and began to increase between day 9 and 17 post-transplantation. Full chimerism was attained in all recipients within 3 days of the initial sustained rise in WBC count.

Patient Clinical Course and Follow-up Monitoring

No patients had episodes of rejection, but patients 1 and 2 had gastrointestinal biopsy proven graft vs. host disease. Patient 2 died with severe graft vs. host disease 3 months post-transplantation, and patient 4 became a mixed chimera 2 months after transplantation without documented relapse and continued to be mixed at the time of this report. The remainder of the patients continue to be full chimeras, with follow-up ranging from 5 to 10 months. Patient 3 relapsed 5 months post-transplantation and was treated with interferon alpha. Since no sample was obtained for STR monitoring during relapse, it is not known whether he became a mixed chimera or remained full. He regained remission while on interferon as documented by morphologic and flow cytometric studies on bone marrow which agreed with the finding of full chimerism by STR.

DISCUSSION

Molecular techniques have been used to monitor engraftment post-BMT using restriction fragment length polymorphisms (RFLP) [7–9], VNTR [2,3,5,10–13], or various combinations of VNTR, STR, cytogenetics, and Y chromosome-specific PCR [14–17]. Each technique has advantages and disadvantages. Y chromosome-specific PCR techniques are very sensitive, but are useful only with different sex donor/recipient pairs [18]. RFLP detection by Southern hybridization has a sensitivity of 1–10% DNA and requires approximately 10^6 cells [7]. The advantages of VNTR over RFLP were demonstrated in a study evaluating chimerism after BMT in 13 patients [4]. VNTR required less DNA (approximately 250 ng); there was no need for DNA digestion, and VNTR was more rapid and sensitive [4]. The sensitivity of VNTR has been documented to be between 1% [3] and 0.1% DNA [4] using different probes. The sensitivity of our

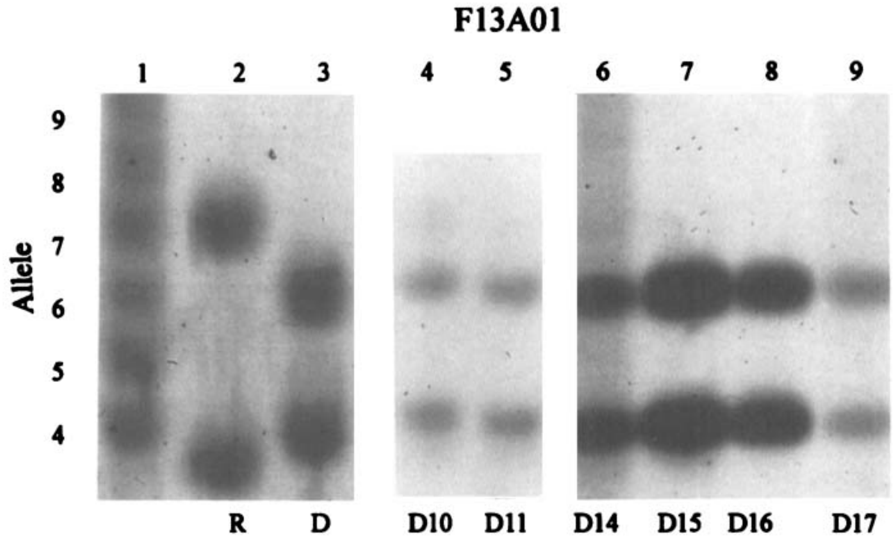

Fig. 2. Documentation of engraftment in patient 2. The ladder of alleles for the F13A01 locus is in lane 1. The pre-transplantation recipient (R) and donor (D) alleles are in lanes 2 and 3, respectively. Lanes 4–9 show recipient alleles post-transplantation days 10, 11, 14, 15, 16, and 17. On days 10, 11, 14, and 15 both donor and recipient alleles are present, while on days 16 and 17 only donor alleles are present (allele 4 with a deletion and 7 are absent) indicating full chimerism.

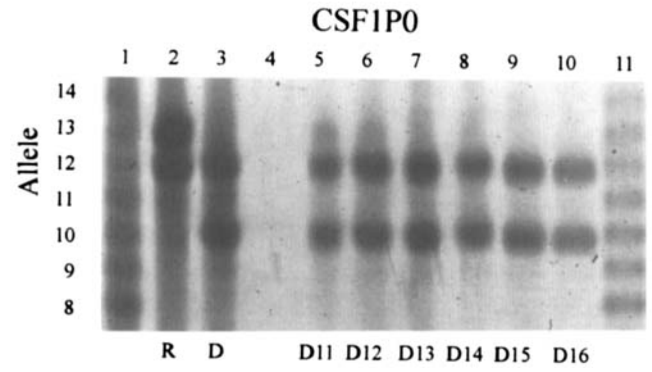

Fig. 3. Documentation of engraftment in patient 3. The ladder of alleles for the CSF1P0 locus is in lanes 1 and 11. The pre-transplantation recipient (R) and donor (D) alleles are in lanes 2 and 3, respectively. Lanes 5–10 show recipient alleles post-transplantation days 11–16. On days 11–13 both donor and recipient alleles are present, while on days 14–16 only donor alleles are present (allele 13 is absent) indicating full chimerism.

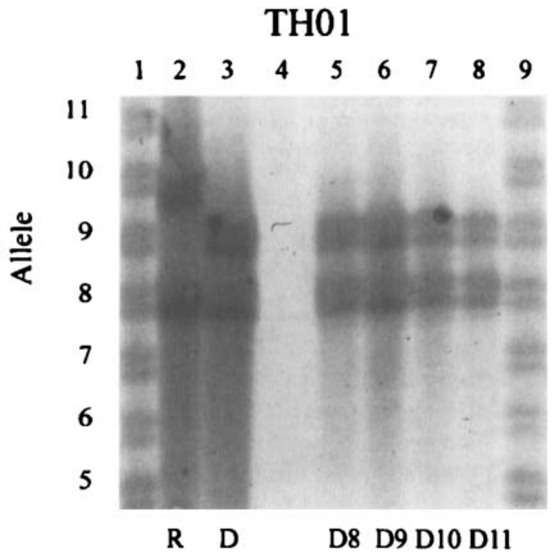

Fig. 4. Documentation of engraftment in patient 4. The ladder of alleles for the TH01 locus is in lanes 1 and 9. The pre-transplantation recipient (R) and donor (D) alleles are in lanes 2 and 3, respectively. Lanes 5–8 show recipient alleles post-transplantation days 8–11. On day 8 both donor and recipient alleles are present, while on days 9–11 only donor alleles are present (allele 10 is absent) indicating full chimerism.

STR technique was comparable (0.05–1%, depending on the probes used) to that reported for VNTR, but the amount of the DNA used (25 ng) was much less. Ideally, a probe should be both informative as well as sensitive. It is possible that a particular probe may delineate earlier or later complete chimerism. Efforts were made when the two most informative probes were chosen, that one probe was more sensitive than the other. Since STR sequences are shorter than VNTR, there is a lower likelihood of interference by degraded DNA or preferential amplification [19–21]. Therefore, STR offers similar sensitivity and fewer potential problems than VNTR.

Documenting early chimeric status may be helpful in assessing the development of graft-vs. host disease, graft rejection, and disease relapse. Early chimerism was demonstrated post-BMT by VNTR in a recent study [2]. Seven of ten donor/recipient pairs were differentiated by the Apoprotein B locus; 4 of the patients became full chime-

TABLE II. Recipients, Type of Transplant, and Date of Engraftment in Relationship With the Initial WBC Recovery*

Recipient	Age	Disease	Transplant	Full chimerism (day posttransplant)	WBC rise (day posttransplant)	Follow-up month
1	34	NHL	PBSCT	9–14	9	10
2	52	AML	BMT	16	17	3
3	41	ALL	BMT	14	12	8
4	45	NHL	PBSCT	9	9	5
5	50	MM	BMT	14	13	5

*NHL = Non-Hodgkin's lymphoma; AML = acute myelogenous leukemia; ALL = acute lymphoblastic leukemia; MM = multiple myeloma; PBSCT = peripheral blood stem cell transplant; BMT = bone marrow transplant.

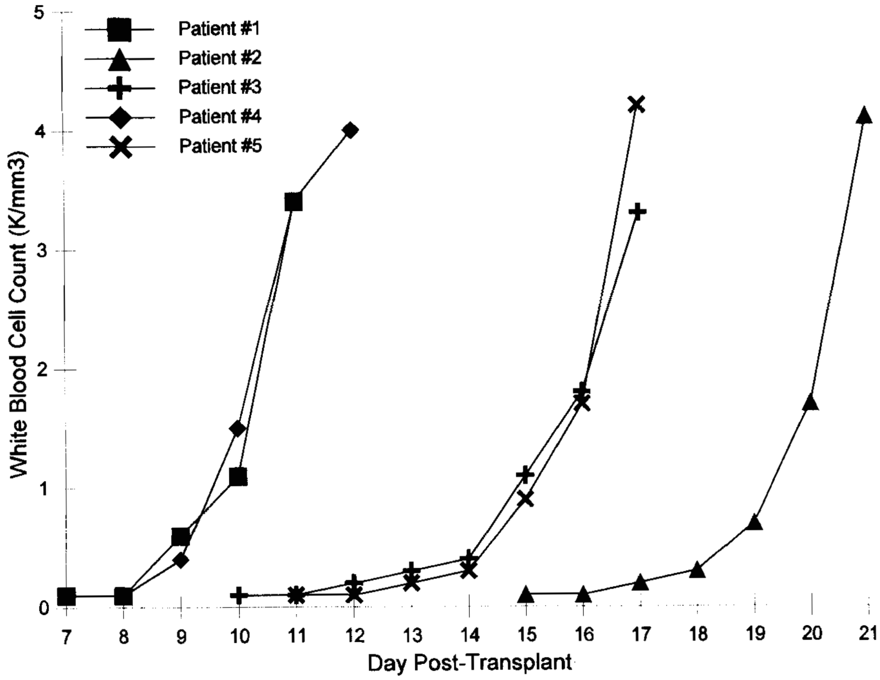

Fig. 5. Early post-transplantation white blood cell (WBC) counts in patients 1–5. The rise if WBC counts coincided with PCR-STR documentation of engraftment. The two PBSCT patients (patient 1 and 4) tended to have an earlier recovery than the BMT patients.

ras between day 8 and 14; 2 remained mixed chimeras, and the chimeric state could not be determined in 1 patient due to shared alleles. In our study, all recipients initially attained full chimerism. At the time of this report, the patient (4) who subsequently reverted to mixed chimerism remains disease free 5 months after transplantation.

Hematopoietic reconstitution is thought to be more rapid in PBSCT than BMT recipients, as evidenced by a faster recovery of peripheral blood counts [22], but this has not previously been documented using molecular methods. In our study, PCR-STR showed that engraftment occurred earlier in our PBSCT patients (9 days and between 9–14 days vs. 14–16 days for BMT patients). Further study with additional patients is necessary to validate this finding.

In our study, the peripheral blood WBC count rose within 3 days of the onset of complete chimerism in all patients, indicating that a sustained rise in WBC count may be a reliable marker of engraftment. Past studies have inferred engraftment from a rising WBC count [23]. However, recipients who remain mixed chimeras also show increases in the WBC count, and therefore more definitive methods such a STR are still desirable to detect the state of chimerism.

Studies using molecular techniques to relate the presence of mixed chimerism to relapse have had contradictory results [10,12,14,16]. One study evaluated 32 pediatric patients by VNTR, and found 5 patients to be stable mixed chimeras for up to 1 year with no increased risk of recurrent disease [11]. A second study with 20 patients

monitored by VNTR and Y chromosome probes demonstrated that 5 patients remained mixed chimeras with no relapse during 84 months of follow-up [14]. Contrasting results were obtained from 32 chronic myelogenous leukemia pateitns who received mainly T cell depleted BMT (30/32) and were evaluated with VNTR. Twenty-two patients had mixed chimerism, and there was a correlation between mixed chimerism and minimal residual disease as determined by PCR for BCR-ABL as well as with cytogenetic and hematologic relapse [10]. When methods such as dinucleotide repeats, Y chromosome-specific PCR, and cytogenetics were used, sustained mixed chimerism itself was not indicative of relapse, but a sudden appearance of mixed chimerism from a previously complete chimerism indicated early graft rejection or disease relapse [16]. Another group of investigators showed that an increase in the proportion of residual recipient cells predicted early relapse. Using fluorescence-tagged primers, they amplified and quantitated PCR DNA products with a DNA sequencer [17]. While the sensitivity of this method is comparable to that of silver stained gels (1%), the ability to quantitate the residual recipient DNA may provide more information in predicting early relapse in certain patients. Especially in patient 4 who became a nonrelapsed mixed chimera, it may be of interest to see, by quantitating the percentage of donor and recipient cells, whether this represents recipient stem cell recovery, or a gradual loss of donor cells.

The molecular techniques used in these studies have different sensitivities which determine the level at which recipient DNA and mixed chimerism is identified. Therefore, some contradictory conclusions in these studies can be explained by different techniques for monitoring chimerism. One recent study comparing relapse rates using VNTR (1% sensitive) to Y chromosome-specific PCR (0.01% sensitive) concluded that the detection of recipient cells above the 1% level might be predictive of relapse while detection of lower levels of recipient DNA is unrelated to outcome [15]. It has also been suggested that differences among studies may be due to different recipient diseases [24].

One area of concern with this highly sensitive PCR-STR technique is that it will detect extraneous DNA. No contaminating bands were identified in this study. It has been suggested that in multiply transfused BMT recipients, transfusions may cause extraneous bands [16]. No third party bands have been documented with engraftment analysis by VNTR [2,3] or STR [25]. We have previously shown that in 3 BMT recipients, no red blood cell unit-specific alleles were detected when peripheral blood was monitored at various time intervals (>30 min) after each of 14 transfusions [25].

PCR-STR is relatively rapid and simple to perform with equipment available in most molecular laboratories. All 15 donor/recipient pairs in this study were differentiated by a least 2 of the probes. It is a cost and time efficient method since the same techniques are used for the detection of all 8 probes. This will limit the necessity of using a combination of molecular markers to find informative loci. The allelic ladders have been well characterized and the probes well studied [26]. For HLA identical, same sex, related donor/recipient pairs, it is one of the most sensitive methods for detecting early engraftment. PCR-STR is also useful in detecting residual host DNA and for monitoring the continued state of recipient chimerism which may help predict clinical course.

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